Journal of Gastroenterology and Hepatology (2003) 18, 1272–1282

STEATOHEPATITIS

Rodent nutritional model of non-alcoholic steatohepatitis: Species, strain and sex difference studies

RICHARD KIRSCH,*,† VIVIAN CLARKSON,* ENID G SHEPHARD,† DAVID A MARAIS,‡ MOHAMED A JAFFER,§ VIVIENNE E WOODBURNE,† RALPH E KIRSCH† AND PAULINE DE LA M HALL*

**Department of Anatomical Pathology, †Medical Research Council and University of Cape Town Liver Research Center, ‡Cape Heart Center and Medical Research Council Cape Heart Group, and §Electron Microscope Unit, University of Cape Town, Cape Town, Western Cape, South Africa*

Abstract

Background and Aim: The methionine choline-deficient (MCD) diet leads to steatohepatitis in rodents. The aim of the present study was to investigate species, strain and sex differences in this nutritional model of non-alcoholic steatohepatitis (NASH).

Methods: Male and female Wistar, Long–Evans and Sprague–Dawley rats, and C57/BL6 mice ($n = 6$ per group) were fed a MCD diet for 4 weeks. Control groups received an identical diet supplemented with choline bitartrate (0.2% w/w) and methionine (0.3% w/w). Liver pathology (steatosis and inflammation) and ultrastructure, liver lipid profile (total lipids, triglycerides, lipid peroxidation products), liver : body mass ratios and serum alanine aminotransferase (ALT) levels were compared between these groups.

Results: The MCD diet-fed male rats developed greater steatosis ($P < 0.001$), had higher liver lipid content ($P < 0.05$) and had higher serum ALT levels ($P < 0.005$) than did female rats. Wistar rats (both sexes) had higher liver lipid levels ($P < 0.05$), serum ALT levels ($P < 0.05$), and liver mass : body mass ratios ($P < 0.025$) than did Long–Evans and Sprague–Dawley rats. In female groups, Wistar rats showed greater fatty change than did the other two strains ($P < 0.05$). All rats fed the MCD diet developed hepatic steatosis, but necrosis and inflammation were minor features and fibrosis was absent. Compared with Wistar rats, male C57/BL6 mice showed a marked increase in inflammatory foci ($P < 0.001$), end products of lipid peroxidation (free thiobarbituric acid reactive substances) ($P < 0.005$), and mitochondrial injury, while showing less steatosis ($P < 0.005$), lower hepatic triglyceride levels, ($P < 0.005$) and lower early lipid peroxidation products (conjugated dienes and lipid hydroperoxides; $P < 0.005$ and $P < 0.01$, respectively).

Conclusions: The Wistar strain and the male sex are associated with the greatest degree of steatosis in rats subjected to the MCD diet. Of the groups studied, male C57/BL6 mice develop the most inflammation and necrosis, lipid peroxidation, and ultrastructural injury, and best approximate the histological features of NASH.

Key words: choline, lipid, lipid peroxidation, methionine, mice, mitochondria, non-alcoholic steatohepatitis, rats, sex, strain.

INTRODUCTION

Non-alcoholic fatty liver disease is one of the common forms of liver disease seen in outpatient practice.[1,2] It is associated with a wide spectrum of morphological manifestations, including fatty change (steatosis), inflammation, necrosis, and occasionally cirrhosis. Overlap between these patterns of injury often exists, and steatosis is frequently associated with hepatocyte injury and inflammation; a condition known as non-alcoholic steatohepatitis (NASH).[3,4]

Until recently, NASH was regarded as a mild disease that rarely progresses to cirrhosis.[3,5,6] However, it is now recognized that a significant proportion of

Correspondence: Dr Richard Kirsch, Department of Anatomical Pathology, Faculty of Health Sciences, University of Cape Town, Observatory 7925, South Africa. Email: rich@chempath.uct.ac.za

Accepted for publication 5 March 2003.

patients with NASH do develop progressive fibrosis leading to cirrhosis, and occasionally, hepatocellular carcinoma.[7–9] Non-alcoholic steatohepatitis is seen in association with obesity, type 2 diabetes mellitus, hyperlipidemia, rapid weight loss and jejunoileal bypass surgery for obesity;[10] a similar pattern of liver injury can occur following exposure to certain drugs and industrial toxins.[11]

Animal models have greatly contributed to the understanding of NASH. While several models of steatosis exist,[12] fewer models of steatohepatitis are available. One such model is the high-fat methionine choline-deficient diet (MCD),[13,14] which results in hepatic steatosis, necroinflammation, and, in the longer term, fibrosis.[14] Although initially established as a model of fatty liver transplantation,[13] its application to the study of NASH pathogenesis has provided important new insights.[14–20] Steatohepatitis in this model is associated with upregulated hepatocyte microsomal cytochrome P450 2E1 (CYP2E1) activity and increased hepatic lipid peroxidation.[14,15] CYP4A proteins provide an alternative source of microsomal pro-oxidant activity in CYP2E1 nullizygous mice fed the MCD diet.[15] The importance of these findings is highlighted by the subsequent demonstration of enhanced CYP2E1 expression,[21] as well as by by-products of oxidative stress[22] in liver biopsies from patients with NASH. Inflammation and fibrosis in the MCD model is leptin-dependent,[16] suggesting that leptin may play a regulatory role in hepatic fibrogenesis in NASH. Recent studies suggest that peroxisome proliferation activator receptor (PPAR)-α-regulated pathways may protect against the development of steatohepatitis, because PPAR-α agonists can prevent steatosis and inflammation in animals fed a high-fat MCD diet.[17]

To date there have been no systematic studies that have investigated the influence of species, strain and sex differences on the degree of hepatic steatosis, inflammation and fibrosis in the MCD nutritional model of NASH. Previous studies have used male Wistar,[14,19] ACI,[13] and Sprague–Dawley rats,[18] male C57/BL6 mice,[15,17,20] and genetically obese male (ob/ob) mice.[16] In humans, genetic factors[23] and sex differences[4,24–27] are known to influence individual susceptibility to NASH and the female sex is an independent predictor of fibrotic severity in NASH.[24] The present study thus sought to further characterize this model by testing the hypothesis that species, strain and sex differences influence liver injury in the MCD nutritional model of NASH.

METHODS

The present study was approved by the Animal Research Review Committee of the Faculty of Medicine, University of Cape Town, South Africa.

Materials

The pelleted MCD diet and the MCD diet supplemented with choline bitartrate (2 g/kg) and DL-methionine (3 g/kg) were custom-made by ICN Biomedicals (Irvine, CA, USA). Chloroform, methanol and glutaraldehyde were obtained from Merck (Darmstadt, Germany). Osmium tetraoxide was from Next Chimica (Centurion, South Africa). Spurr's resin was obtained from Agar Scientific limited (Stanstead, UK). All other materials were obtained from Sigma (St Louis, MO, USA).

Animals and diet

Wistar rats were obtained from the Medical Research Council Animal Unit (Cape Town, South Africa), Long–Evans rats and C57/BL6 mice from the University of Cape Town Animal Unit, and Sprague–Dawley rats from the University of Witwatersrand Animal Unit (Johannesburg, South Africa). All animals were 12 weeks of age at the commencement of the study. Animals were allowed to acclimatize to their new conditions for 1 week prior to the commencement of the study. The eight study groups, comprising male and female rats of the three different rat strains and C57/BL6 mice ($n = 6$ per group) were fed the MCD diet for 4 weeks, while control groups ($n = 6$ per group) received an identical diet to which choline bitartrate (2 g/kg) and DL-methionine (3 g/kg) was added. An additional control group was included that received standard rodent food. Rats were given free access to food and water and were weighed at weekly intervals for the duration of the study. The duration of the study and the composition of the diets were based on those used in previous studies.[14–20]

Tissue preparation and analysis

At the end of the study, animals were ether anesthetized, killed, and their livers were removed and weighed and sampled for histological assessment. Additional samples were snap frozen in liquid nitrogen for later analysis of liver lipid levels and lipid peroxidation. Samples for electron microscopy were fixed in 2.5% glutaraldehyde in phosphate buffered saline (pH = 7.4).

Biochemical analyses

Serum was assayed for alanine aminotransferase (ALT) activity spectrophotometrically as described by Hørder and Rej.[28] Frozen liver samples were thawed on ice, homogenized and sonicated with chilled SEAP buffer (0.15 mol/L NaCl, 0.001 mol/L EDTA, 0.01 g% NaN_3, 0.002 mol/L NaH_2PO_4, pH = 7.4), and brought to a final volume of 2 mL, of which 800 μL was used for lipid extraction (triglycerides, conjugated dienes and lipid hydroperoxides) and the remainder for other assays. Lipid extraction was performed according to Folch,[29] as modified by Bligh and Dyer,[30] and extracts were dried under nitrogen jets. Triglycerides were measured colorimetrically in lipid extracts using a commercial kit (Roche, Man-

nheim, Germany) as previously reported.[31] Early, intermediate and late products of lipid peroxidation (conjugated dienes, lipid hydroperoxides and thiobarbituric acid reactive substances [TBARS], respectively) were measured by spectrophotometric assays. Conjugated dienes and lipid hydroperoxides were measured in liver lipid extracts as described by Vasankari *et al.*[32] and Jziang *et al.*[33], respectively. Free and total TBARS in liver homogenates were measured by the method described by Asakawa *et al.*[34] Assays were performed both in the presence and absence of the antioxidant butylated hydroxytoluene (BHT); BHT limits the generation of TBARS during the assay, ensuring that only the products present in the liver at the time of death are measured (free TBARS).[34] Protein concentrations in liver homogenates were measured using the Markwell modification of the Lowry assay.[35] Hepatic lipid indices were expressed per milligram of protein.

Histopathology

Sections of formalin-fixed, paraffin-embedded samples were stained with H&E and analyzed using light microscopy. Selected samples were stained with Sirius red. Fatty change was graded according to the percentage of hepatocytes containing macrovesicular fat (grade 1: 0–25%; grade 2: 26–50%; grade 3: 51–75%; grade 4, 76–100%). Necroinflammation was quantified histologically by counting inflammatory foci, arbitrarily defined as groups of five or more leukocytes in 20 consecutive high-power fields (×40 objective). Assessment of liver injury was performed using coded slides to avoid observer bias.

Electron microscopy

For ultrastructural analysis, 1 mm^3 samples were fixed in glutaraldehyde (2.5%), postfixed in osmium tetraox-

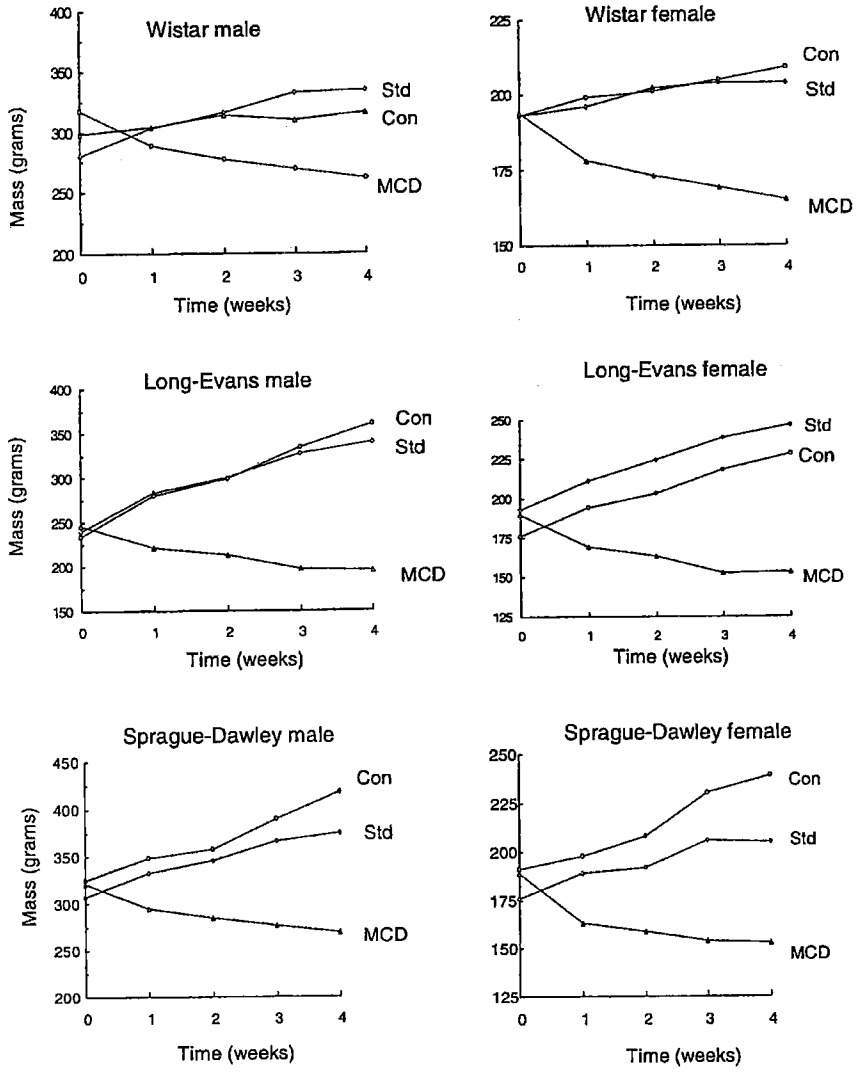

Figure 1 Body masses of rats fed methionine choline-deficient (MCD) diet, control (MCD supplemented with choline [2 g/kg] and methionine [3 g/kg]), and standard diets. Data are expressed as the mean masses of the six animals in each group.

ide (2%) and embedded in Spurr's resin. Sections were stained with uranyl acetate (2%, w/v) and lead citrate prior to analysis with electron microscopy (Zeiss EM 109; Oberkochen, Germany). For quantification of mitochondrial size, five random fields (magnification ×20 000) from five random cells in each section were photographed by an electron microscopist blinded to treatment groups; each photograph contained 5–10 mitochondria in a particular cell. Morphometric measurement of the mitochondrial area was performed on the five largest mitochondria per photograph using the JLGenias image analysis software (Joyce-Loebl, Gateshead, UK).

Statistical analysis

Data was subjected to statistical analysis using the Kruskal–Wallis test and the Student's *t*-test where applicable.

RESULTS

Rat study: Strain and sex differences

Animal growth

In all groups, animals fed the MCD diet lost weight compared with those fed control or standard rodent diets; the latter showed a steady increase in weight (Fig. 1). Apart from weight loss, the general condition of the animals remained satisfactory.

Liver mass and morphology

The livers of male and female Wistar rats fed the MCD diet increased in mass relative to controls, while those from male and female Long–Evans and Sprague–Dawley rats decreased in mass (Table 1). To control for the loss of body mass induced by the MCD diet, liver mass was expressed as a percentage of body mass (relative liver mass). Male and female Wistar rats fed the MCD diet showed marked increases in relative liver mass while minimal changes were seen in MCD-fed Long–Evans and Sprague–Dawley rats ($P < 0.0025$). Macroscopically, livers from Wistar rats fed the MCD diet appeared pale and fatty compared with controls; these changes were less noticeable in MCD-diet-fed Long–Evans and Sprague–Dawley rats (Fig. 2).

Serum alanine aminotransferase levels

Serum ALT levels were significantly higher in all groups fed the MCD diet compared with controls ($P < 0.05$), except in the case of female Sprague–Dawley rats (Fig. 3). Wistar rats fed the MCD diet (male and female) had higher ALT levels than did their counterparts from other strains ($P < 0.01$ and $P < 0.05$, respectively). As a group, MCD-diet-fed male rats had higher ALT levels than their female counterparts ($P < 0.02$).

With respect to liver weights, liver histology and serum transaminase levels, there were no differences

Table 1 Liver mass (absolute and relative†) and changes in relative liver mass resulting from the methionine choline-deficient (MCD) diet (relative liver mass [MCD]/relative liver mass [control] × 100) in the six study groups

	MCD						Control					
	Wistar		Long–Evans		Sprague–Dawley		Wistar		Long–Evans		Sprague–Dawley	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Liver mass (grams)	12.50 ± 3.16	8.63 ± 0.62	7.88 ± 1.64	4.95 ± 1.28	10.03 ± 1.20	4.87 ± 0.42	10.40 ± 2.51	6.42 ± 0.55	13.00 ± 1.64	7.40 ± 0.77	14.10 ± 1.79	7.70 ± 0.63
Relative liver mass (liver mass as a percentage of body mass)	4.73 ± 0.97	5.23 ± 0.28	4.03 ± 0.75	3.20 ± 0.68	3.72 ± 0.26	3.20 ± 0.30	3.27 ± 0.21	3.05 ± 0.22	3.60 ± 0.42	3.30 ± 0.55	3.30 ± 0.45	3.25 ± 0.22
Percentage change in relative liver mass	45	72	12	0	12	0	NA	NA	NA	NA	NA	NA

†Relative liver mass = liver mass as a percentage of body mass. Data are expressed as mean ± SD for each group. NA, not applicable.

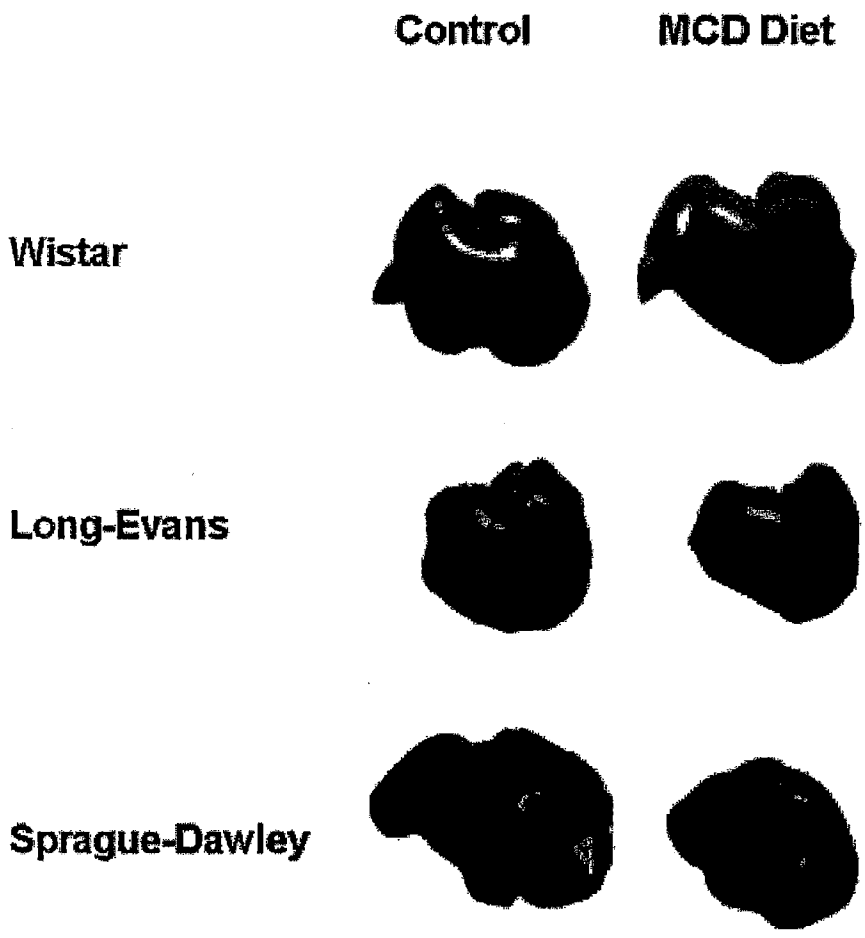

Figure 2 Macroscopic appearance of livers from male Wistar, Long–Evans and Sprague–Dawley rats fed control or methionine choline-deficient (MCD) diets. Female counterparts showed similar macroscopic features.

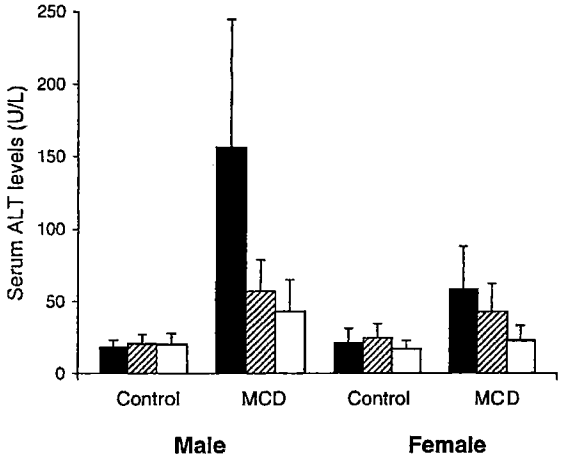

Figure 3 Serum alanine aminotransferase (ALT) levels in male and female (■) Wistar, (▨) Long–Evans and (□) Sprague–Dawley rats fed methionine choline-deficient (MCD) or control diets for 4 weeks. Data are expressed as mean ± SD for each group.

between animals fed the control diet (MCD diet plus choline bitartrate [2 g/kg] and DL-methionine [3 g/kg]) and those receiving standard rodent diet (data not shown). Therefore, only the data for the group receiving the control diet is presented.

Liver lipid content

The liver lipid content (expressed as mg lipid/100 mg liver weight) was higher in male and female Wistar rats fed the MCD diet than in their counterparts in the other two strains ($P < 0.05$ and $P < 0.02$, respectively) (Fig. 4). As a group, male rats had higher liver lipid levels than female rats ($P < 0.002$).

Liver pathology

Livers from animals fed the MCD diet showed macrovesicular steatosis that varied in quantity and distribution in the various groups (Fig. 5). In the least affected groups (female Long–Evans and Sprague–Dawley rats) steatosis was confined to zone 3, while in the most severely affected animals (male Wistar rats)

close to 100% of hepatocytes showed macrovesicular steatosis. When steatosis was graded according to the percentage of hepatocytes containing macrovesicular fat, Wistar rats had higher scores than did the other strains, although this was only statistically significant in female rats ($P < 0.05$) (Fig. 6). As a group, male rats showed a greater degree of steatosis than did female rats ($P < 0.0002$). In all rats fed the MCD diet, inflammation and necrosis were minor features, while fibrosis was absent.

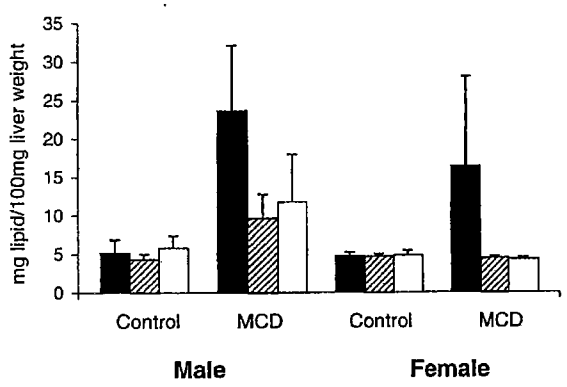

Figure 4 Total liver lipid content (mg/100 mg liver) in male and female (■) Wistar, (▨) Long–Evans and (□) Sprague–Dawley rats fed methionine choline-deficient (MCD) or control diets for 4 weeks. Data are expressed as mean ± SD for each group.

Mouse study: Comparison of C57/BL6 mice and Wistar rats

Male and female C57/BL6 mice fed the MCD diet were analyzed with respect to serum ALT levels, liver histology (steatosis and necroinflammation), ultrastructure and lipid biochemistry and compared with Wistar rats, the most susceptible rat strain.

Male C57/BL6 mice showed a marked increase in necroinflammatory foci compared with female C57/BL6 mice ($P < 0.02$) and Wistar rats of either sex ($P < 0.001$) (Table 2, Fig. 7a,b). These necroinflammatory foci were composed predominantly of lymphocytes with occasional neutrophils. Hepatocyte ballooning and Mallory bodies were not present. In some mice, focal pericellular fibrosis was noted (Fig. 7c). Both male and female mice showed less steatosis than did the Wistar

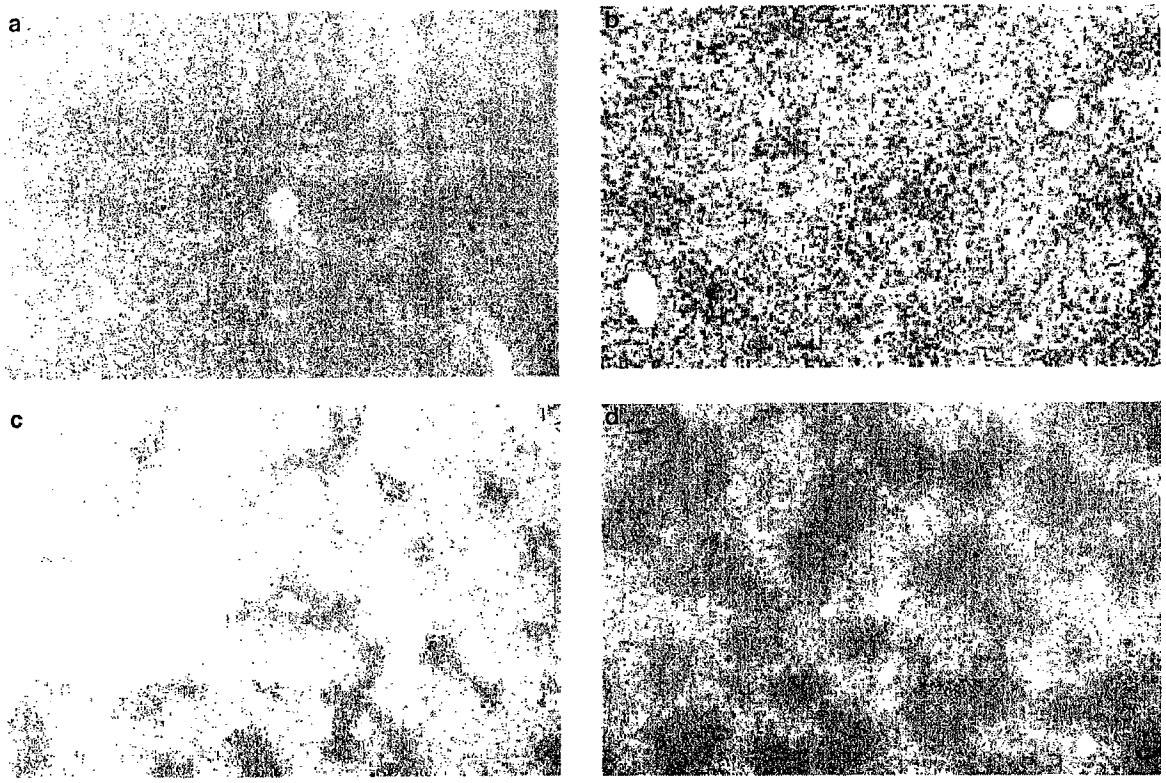

Figure 5 HE-stained sections from rats fed the methionine choline-deficient (MCD) or control diets for 4 weeks, illustrating differences in fatty change. (a) Control rat (magnification ×40). The histological appearance is normal and representative of controls from all groups. (b) A male Wistar rat fed the MCD diet. Fatty change involves the entire liver lobule. (c) Female Wistar rat fed the MCD diet. Fatty change involves zones 3 and 2 with periportal sparing. (d) Female Long–Evans rat fed the MCD diet. Fatty change is largely confined to zone 3.

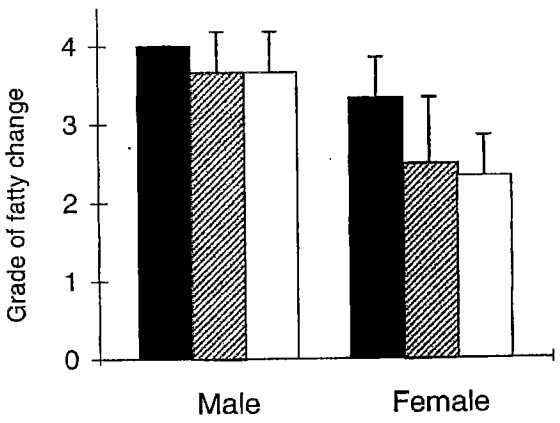

Figure 6 Average histological grade of fatty change in male and female (■) Wistar, (▨) Long–Evans and (□) Sprague–Dawley rats fed the methionine choline-deficient diet for 4 weeks. Fatty change was graded histologically according to the percentage of hepatocytes containing macrovesicular fat (grade 1: 0–25%; grade 2: 26–50%; grade 3: 51–75%; grade 4, 76–100%). Data are expressed as mean ± SD for each group.

rats ($P < 0.005$). Serum ALT levels were elevated in male and female MCD-fed mice compared with controls ($P < 0.005$); however, the differences between male and female rodents and between C57/BL6 mice and Wistar rats were not statistically significant (Table 2).

Male C57/BL6 mice and Wistar rats were further studied with respect to hepatic ultrastructural morphology and lipid peroxidation; female rodents were not included in these analyses because of the minor degree of liver injury seen in these animals.

Ultrastructural morphology

Male C57/BL6 mice and Wistar rats fed the MCD diet showed evidence of ultrastructural injury that was not observed in controls; this was more pronounced in C57/BL6 mice. Such changes included profound mitochondrial swelling, proliferation of the smooth endoplasmic reticulum, loss of the normal relationship between the rough endoplasmic reticulum and mitochondria, and blebbing of the nuclear membrane (Fig. 7d and 8). In MCD-diet-fed mice, most cells contained a number of enlarged mitochondria, some of which were 3–4-fold the normal size; no such mitochondria were noted in control animals. Morphometric analysis of confirmed differences in mitochondrial size in MCD-diet-fed and control mice (average mitochondrial surface area: control $0.38 \pm 0.12\ \mu m^2$ *vs* MCD $0.71 \pm 0.16\ \mu m^2$; $P < 0.03$). Paracrystalline inclusions, reported in the mitochondria of humans[36] and animals[20] with NASH, were not seen in the present study.

Male C57/BL6 mice and Wistar rats were also compared with respect to hepatic lipid biochemistry. Triglyceride levels were far higher in MCD-diet-fed Wistar rats compared with their murine counterparts ($P < 0.005$) (Table 3). The MCD-diet-fed rodents

Table 2 Average histological grades of fatty change, inflammatory activity, and serum alanine aminotransferase (ALT) levels in male and female Wistar and C57/BL6 mice fed methionine choline-deficient (MCD) and control diets for 4 weeks

	MCD				Control			
	Wistar		C57/BL6		Wistar		C57/BL6	
	Male	Female	Male	Female	Male	Female	Male	Female
Inflammatory foci† per 20 high-power field	5.00 ± 3.29	6.50 ± 5.13	41.67 ± 25.58	6.67 ± 0.82	0.67 ± 0.82	0.17 ± 0.41	2.75 ± 1.26	2.33 ± 1.97
Fatty change (average histological grade)	4.00 ± 0.00	3.3 ± 0.52	2.67 ± 0.52	2.33 ± 0.82	NA	NA	NA	NA
Serum ALT levels (IU/L)	156.67 ± 29.62	58.67 ± 29.62	99.83 ± 36.76	70.67 ± 16.17	18.83 ± 4.62	21.50 ± 9.71	18.67 ± 6.77	21.33 ± 12.23

†Inflammatory focus defined arbitrarily as a collection of ≥5 leukocytes. Data are expressed as mean ± SD for each group. NA, not applicable.

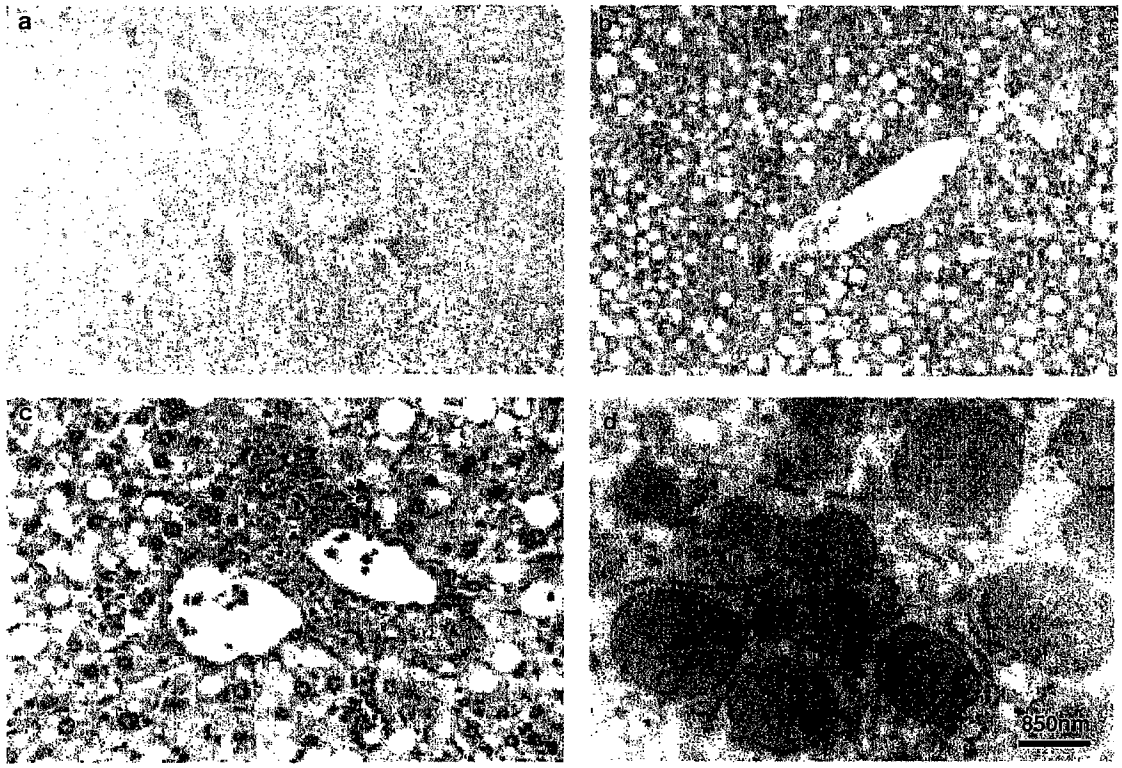

Figure 7 (a,b) HE-stained sections from the liver of a male C57/BL6 mouse fed the methionine choline-deficient (MCD) diet for 4 weeks (×40 and ×200 magnification, respectively) illustrating mild steatosis but extensive necroinflammation. (c) Bile Sirius red stained sections illustrating pericellular fibrosis in an MCD-diet-fed male C57/BL6 mouse. Collagen is stained red. (d) Representative transmission electron micrograph illustrating mitochondrial enlargement in a male C57/BL6 mouse fed the MCD diet (compare with control mitochondria, Fig. 8a).

showed elevated levels of early, intermediate and late products of lipid peroxidation compared with their respective controls (Table 3). The MCD-diet-fed rats had higher levels of conjugated dienes and lipid hydroperoxides (early and intermediate products of lipid peroxidation) than did mice ($P < 0.005$ and $P < 0.01$, respectively); in contrast, mice showed far greater levels of free TBARS (end-products of lipid peroxidation) than did rats ($P < 0.005$). Total TBARS, which reflect merely the total available peroxidizable lipid, were more elevated in rats, in keeping with elevated triglyceride levels in these animals.

DISCUSSION

The present study has identified species, strain and sex differences in the MCD rodent nutritional model of NASH. Among the rat strains studied, male Wistars developed the greatest degree of steatosis when fed the MCD diet; in all rats inflammation and necrosis were minor features and fibrosis was absent. In contrast, male C57/BL6 mice fed the MCD diet showed florid hepatic necroinflammation with a lesser degree of steatosis. Consistent with previous studies, all animals fed the MCD diet lost weight, while controls showed a steady increase in body weight.[13,14]

The increased susceptibility of male rodents to liver injury in this nutritional model of NASH differs from human NASH, in which women show both a higher prevalence of NASH and increased fibrotic activity.[3,24–27] Biochemical mechanisms unique to the MCD model may be responsible for the sex differences observed in the present study. The proposed biochemical basis for steatosis in this model is a block in phosphatidylcholine (PC) synthesis resulting from a dietary deficiency in methionine and choline.[37] In animals and in humans, PC synthesis may occur via two pathways: one that involves the direct incorporation of preformed choline into phosphatidyl compounds, and the other that is dependent upon the stepwise methylation of phosphatidylethanolamine by *S*-adenosyl methionine.[38–40] Both pathways of PC synthesis are thus blocked by a dietary deficiency in choline and methionine. Because PC is an essential component of very-low-density lipoprotein, which is responsible for triglyceride clearance from hepatocytes, PC deficiency leads to the accumulation of triglycerides within hepatocytes, and thus steatosis.[37] Several studies have demonstrated sex differences in the

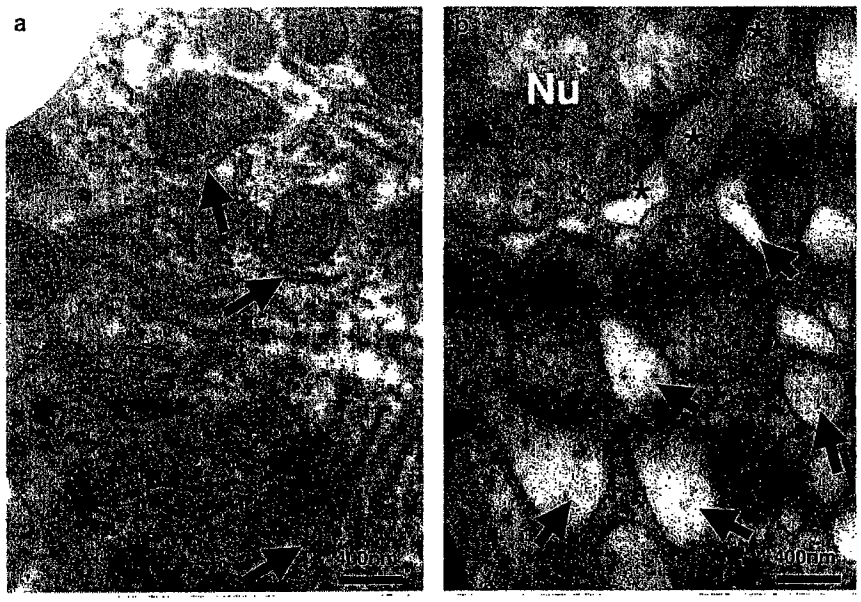

Figure 8 (a) Transmission electron micrograph of a representative hepatocyte from a control animal showing normal-sized mitochondria surrounded by irregularly disposed cysternae of rough endoplasmic reticulum (arrows). Smooth endoplasmic reticulum is not prominent. (b) Transmission electron micrograph of a representative hepatocyte from a mouse fed the MCD diet showing proliferation of smooth endoplasmic reticulum (arrows) and loss of the normal relationship between rough endoplasmic reticulum and mitochondria. Blebbing of the nuclear membrane (*) was frequently observed.

Table 3 Hepatic triglycerides and lipid peroxidation products including thiobarbituric acid reactive substances (TBARS, total and free), lipid hydroperoxides and conjugated dienes in male Wistar rats and C57/BL6 mice fed methionine choline-deficient (MCD) or control diets for 4 weeks. Data are expressed as mean ± SD for each group

	Wistar rats		C57/BL6 mice	
	Control	MCD	Control	MCD
Triglycerides (μg/mg protein)	92.7 ± 13.4	558.0 ± 140.0	71.6 ± 18.0	172.0 ± 12.9
TBARS (free) (nmol/mg protein)	0.44 ± 0.12	2.20 ± 1.30	1.30 ± 2.60	11.20 ± 2.70
TBARS (total) (nmol/mg protein)	4.5 ± 0.7	307.1 ± 29.1	5.2 ± 1.7	81.9 ± 25.1
Lipid hydroperoxides (nmol/mg protein)	0.53 ± 0.16	8.70 ± 0.70	1.20 ± 1.20	6.60 ± 1.30
Conjugated dienes (μmol/mg protein)	7.11 ± 0.27	27.60 ± 5.50	7.70 ± 3.00	12.12 ± 3.60

pathways of PC synthesis between animals and humans, with methionine-dependent pathways apparently favored by estrogen.[39–41] Female rats fed a choline- (but not methionine-) deficient diet have been shown to develop far less hepatic steatosis than male rats.[40] This difference has been attributed to the greater propensity of females to use the methionine-dependent pathway of PC synthesis.[40] In the MCD model, however, where methionine deficiency is combined with choline deficiency, female rats would be expected to lose this apparent 'advantage', as they can no longer exploit the methionine-dependent pathway of PC synthesis. This was not observed in the present study where female rodents consistently developed less steatosis than male rodents. One possible explanation may be the ability of methionine to undergo regeneration following methylation reactions during PC synthesis. In such reactions, only the methyl group in *S*-adenosyl-methionine is consumed; the remainder of the molecule is intact as *S*-adenosylhomocysteine, which can be hydrolyzed to release free homocysteine. Subsequent methylation of homocysteine results in the regeneration of methionine, with methyl groups being donated by cytosolic 5,10-methylene-H_4-folate.[38] Thus, methionine-dependent pathways of PC synthesis may continue to operate, albeit at a lower level, in the presence of a dietary deficiency of methionine, allowing female rodents to retain their relative advantage over male rodents. Sex differences in metabolic pathways are well recognized in other rodent models of hepatic injury,[42] and in the hepatic metabolism of drugs in animals and humans.[43,44]

Marked strain-related differences in steatosis were also identified in rats fed the MCD, with Wistar rats showing the greatest propensity to develop steatosis. The mechanisms underlying these differences are uncertain. It is possible that subtle variations in PC synthesis, as described above, may exist, or that genetic differences at the level of hepatic free fatty acid (FFA) uptake, endogenous FFA synthesis, or β-oxidation of FFA may play a role. Strain-related differences in metabolic pathways and related liver injury have been demonstrated in other animal models of hepatotoxicity, such as the hypoxic halothane model.[45] Recent studies of ani-

mals with inherited mutations involving genes regulating hepatic carbohydrate and lipid metabolism, and in genetically manipulated mice, suggest that genetic factors may play a role in the pathogenesis of NASH.[12] While the influence of genetic factors in human NASH remains unclear, familial clustering of NASH in kindreds has recently been reported.[23]

The rodent group most profoundly affected by the MCD diet was the male C57/BL6 mice. In contrast to other groups, these animals developed florid necroinflammation and showed some evidence of early perivenular fibrosis. Interestingly, C57/BL6 mice developed less steatosis than did rats, suggesting that the increased necroinflammation is not a function of steatosis, but more likely of downstream events such as lipid peroxidation. Lipid peroxidation can injure hepatocytes both directly and indirectly through the pro-inflammatory and profibrogenic properties of its end-products, malondialdehyde and 4-hydroxynonenal.[14] Indeed, the present study found end-products of lipid peroxidation (free TBARS) to be markedly elevated in male C57/BL6 mice compared with male Wistar rats. Interestingly, early and intermediate products of lipid peroxidation (conjugated dienes and lipid peroxides, respectively) were higher in rats than in mice, and this may reflect the increased availability of lipid substrate in these animals and/or less progression to end-products. Mice, in contrast, have less steatosis and lower levels of early lipid peroxide products, but a greater propensity to take the lipid peroxidation process to completion (as evidenced by the higher free TBARS levels). The total TBARS merely reflect the total lipid available for lipid peroxidation *in vitro* and, in the present study, appear to correlate with the degree of steatosis and hepatic triglyceride levels.

Male C57/BL6 mice showed ultrastructural evidence of hepatocyte injury, including profound mitochondrial enlargement, blebbing of nuclear membranes, and dissociation of rough endoplasmic reticulum from mitochondria. The proliferation of smooth endoplasmic reticulum seen in these animals is consistent with the upregulation of CYP2E1 reported in this model.[14,15] Although ultrastructural changes were also present in MCD-fed Wistar rats, these were less pronounced than in mice. Para-crystalline intramitochondrial inclusions that have been reported in human patients with NASH[36] were not observed in this study; such inclusions have, however, been observed in animals fed the MCD diet for longer periods.[20] Mitochondrial injury in animals fed the MCD diet may be a result oxidative stress and consequent lipid peroxidation of mitochondrial membranes. Mitochondrial injury activates hepatocyte apoptotic mechanisms and, when more severe, results in ATP depletion and hepatocyte necrosis,[46] a prominent feature in male C57/BL6 mice. The increased lipid peroxidation (evidenced by increased free TBARS) in the C57/BL6 mice compared with Wistar rats may, in part, explain differences in necroinflammation in these animals.

In conclusion, the present study has demonstrated profound species, strain and sex differences in the MCD nutritional model of NASH. Of the groups studied, male C57/BL6 mice develop the histological features that most closely resemble those seen in human NASH.

REFERENCES

1 Byron D, Minuk CU. Profile of an urban hospital based practice. *Hepatology* 1996; 24: 813–15.
2 Clark JM, Brancati FL, Diehl AM. Nonalcoholic fatty liver disease. *Gastroenterology* 2002; 122: 1649–57.
3 Ludwig J, Viggiano TR, McGill DB, Ott BJ. Nonalcoholic steatohepatitis. *Mayo Clinic Proc.* 1980; 55: 434–8.
4 Hall P de la M. Alcoholic liver disease. In: MacSween RNM, Burt AD, Portman BC, Ishak KG, Scheuer PJ, Anthony PP, eds. *Pathology of the Liver*, 4th edn. Edinburgh: Churchill Livingstone, 2002; 273–311.
5 Eriksson S, Eriksson KF, Bondesson L. Nonalcoholic steatohepatitis in obesity: a reversible condition. *Acta Med. Scand.* 1986; 220: 83–8.
6 Itoh S, Yougel T, Kawagoe K. Comparison between nonalcoholic steatohepatitis and alcoholic hepatitis. *Am. J. Gastroenterol.* 1987; 82: 650–4.
7 Bacon BR, Farahvash MJ, Janney CG, Neuschwander-Tetri BA. Nonalcoholic steatohepatitis: an expanded clinical entity. *Gastroenterology* 1994; 107: 1103–9.
8 Powell EE, Cooksley WGE, Hanson R, Searle J, Halliday JW, Powell LW. The natural history of nonalcoholic steatohepatitis. a follow-up study of forty-two patients for up to 21 years. *Hepatology* 1990; 11: 74–80.
9 Ludwig J, McGill DB, Lindor KD. Review: nonalcoholic steatohepatitis. *J. Gastroenterol. Hepatol.* 1997; 12: 398–403.
10 Chitturi S, Farrell GC. Etiopathogenesis of nonalcoholic steatohepatitis. *Semin. Liver Dis.* 2001; 21: 89–104.
11 Farrell GC. Drugs and steatohepatitis. *Semin. Liver Dis.* 2002; 22: 185–94.
12 Koteish A, Diehl AM. Animal models of steatosis. *Semin. Liver. Dis.* 2001; 21: 57–70.
13 Teramoto K, Bowers JL, Khettry U, Palombo JD, Clouse ME. A rat fatty liver transplant model. *Transplantation* 1993; 55: 737–41.
14 Weltman MD, Farrell GC, Liddle C. Increased hepatocyte CYP2E1 expression in a rat nutritional model of steatosis with inflammation. *Gastroenterology* 1996; 111: 1645–53.
15 Leclercq IA, Farrell GC, Field J, Bell DR, Gonzales FJ, Robertson GR. Role of CYP2E1 and CYP4A enzymes as microsomal catalysts of lipid peroxides in murine non-alcoholic steatohepatitis. *J. Clin. Invest.* 2000; 105: 1067–75.
16 Leclercq IA, Farrell GC, Schreimer PJ, Robertson GR. Leptin is essential for the hepatic fibrogenic response to chronic liver injury. *J. Hepatol.* 2002; 37: 206–13.
17 Ip E, Robertson G, Hall P, Kirsch R, Farrell G, Leclercq I. Effects of altered lipid metabolism on the development of non-alcoholic steatohepatitis [Abstract]. *Hepatology* 2002; 36: 403A.
18 Pera N, Phung N, Leclercq I, Farrell GC, George J. Oxidative stress and the evolution of hepatic fibrogenesis in a rodent nutritional model of non-alcoholic steatohepatitis [Abstract]. *J. Gastroenterol. Hepatol.* 2001; 16 (Suppl.): A77.

19 Zhang B-H, Weltman M, Farrell GC. Does steatohepatitis impair liver regeneration? A study in a dietary model of non-alcoholic steatohepatitis in rats. *J. Gastroenterol. Hepatol.* 1999; **14**: 133–7.

20 Phung N, Farrell G, Robertson G, George G. Antioxidant therapy with Vitamin E ameliorates fibrosis in MCDD-associated NASH [Abstract]. *J. Gastroenterol. Hepatol.* 2001; **16** (Suppl.): A52.

21 Weltman MD, Farrell GC, Hall P de la M, Ingelman-Sundberg M, Liddle C. Hepatic cytochrome P4502E1 is increased in patients with non-alcoholic steatohepatitis. *Hepatology* 1998; 27: 128–33.

22 Sanyal AJ, Campbell-Sargent C, Mirshahi F *et al.* Nonalcoholic steatohepatitis: Association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* 2001; **120**: 1183–92.

23 Struben VMD, Hespenheide EE, Caldwell SH. Non-alcoholic steatohepatitis and crytogenic cirrhosis within kindreds. *Am. J. Med.* 2000; **108**: 9–13.

24 Angulo P, Keach JC, Batts KP, Lindor KD. Independent predictors of liver fibrosis in patients with non-alcoholic steatohepatitis. *Hepatology* 1999; **30**: 1356–62.

25 Diehl AM, Goodman Z, Ishak KG. Alcohol-like liver disease in non-alcoholics. A clinical and histological comparison with alcohol induced liver injury. *Gastroenterology* 1988; **95**: 1056–62.

26 Powell EE, Cooksley WG, Hanson R. The natural history of non-alcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. *Hepatology* 1990; **11**: 74–80.

27 Lee RG. Non-alcoholic steatohepatitis: a study of 49 patients. *Hum. Pathol.* 1989; **20**: 594–8.

28 Hørder M, Rej R. Alanine transaminase. In: Bergmeyer HU, Bergmeyer J, Grassl M, eds. *Methods of Enzymatic Analysis*, 3rd edn. Weinheim: Verlag-Chemie, 1983; 444–56.

29 Folch J, Lees M, Sloane-Stanley GA. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 1957; **226**: 497–509.

30 Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 1959; **37**: 911–17.

31 McGowan MW, Artiss JD, Strandbergh DR, Zak B. A peroxidase coupled method for the colorimetric determination of serum triglycerides. *Clin. Chem.* 1983; **29**: 538–42.

32 Vasankari T, Urho K, Olli H, Kapanen J, Markku A. Measurement of serum lipid peroxidation during exercise using three different methods: diene conjugation, thiobarbituric acid reactive material and fluorescent chromolipids. *Clin. Chim. Acta* 1995; **234**: 63–9.

33 Jziang ZY, Hunt JV, Wolff SP. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxides in low density lipoprotein. *Anal. Biochem.* 1992; **202**: 384–9.

34 Asakawa T, Matsushita S. Colouring conditions of thiobarbituric acid test for detecting lipid hydroperoxides. *Lipids* 1980; **15**: 137–40.

35 Lowry OH, Rosebrough NJ, Farr AL, Randall RL. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 1951; **193**: 265–76.

36 Caldwell SH, Swerdlow RH, Khan EM *et al.* Mitochondrial abnormalities in non-alcoholic steatohepatitis. *J. Hepatol.* 1999; **31**: 430–4.

37 Ghoshal AK, Farber E. Choline defiency, lipotrope deficiency and the development of liver disease including cancer: a new perspective. *Lab. Invest.* 1993; **68**: 255–60.

38 McGilvery RW, Goldstein GW. Amino acids: one-carbon pool and total balance. In: *Biochemistry: a Functional Approach*, Igaku-Shoin/Saunders International 3rd edn. Japan: WB Saunders, 1983; 628–37.

39 Lindblad L, Schersten T. Incorporation rate in vitro of choline and methyl-methionine into human lecithins. *Scand. J. Gastroenterol.* 1976; **11**: 587–91.

40 Tessitore L, Secca E, Greco M, Pani P, Dianzani MU. Sexually differentiated response to choline in choline deficiency and ethionine intoxication. *Int. J. Exp. Pathol.* 1995; **76**: 125–9.

41 Negishi I, Aizawa Y. Sex difference in the development of fatty liver by orotic acid. *Jpn. J. Pharmacol.* 1975; **25**: 289–94.

42 Plummer JL, Hall P de la M, Jenner MA, Cousins MJ. Sex differences in halothane metabolism and hepatotoxicity in a rat model. *Anaesth. Analg.* 1985; **64**: 563–9.

43 Kato R. Sex-related differences in drug metabolism. *Drug Metab. Rev.* 1974; **3**: 1–32.

44 Wilson K. Sex-related differences in drug disposition in man. *Clin. Pharmacokinet.* 1984; **9**: 189–202.

45 Gourlay GK, Adams JF, Cousins MJ, Hall P de la M. Genetic differences in reductive metabolism and hepatotoxicity of halothane in three rat strains. *Anaesthesiology* 1981; **55**: 96–103.

46 Rashid A, Wu T-C, Huang C-C *et al.* Mitochondrial proteins that regulate apoptosis and necrosis are induced in mouse fatty liver. *Hepatology* 1999; **29**: 1131–8.

Hayward A, Belcher JC, Mendes EN. *Helicobacter bilis* sp. nov., a novel *Helicobacter* isolated from bile, livers, and intestines of aged, inbred mouse strains. J Clin Microbial 1995;33:445–454.
9. Stanley J, Linton D, Burnens AP, Dewhirst FE, Owen RJ, Porter A, On SLW, Costas M. *Helicobacter canis* sp. nov., a new species from dogs: an integrated study of phenotype and genotype. J Gen Microbiol 1993;139:2495–2504.
10. Stanley J, Linton D, Burnens AP, Dewhirst FE, On SL, Porter A, Owen RJ, Costas M. *Helicobacter pullorum* sp. nov.—genotype and phenotype of a new species isolated from poultry and from human patients with gastroenteritis. Microbiology 1994;140: 3441–3449.
11. Fox JG, Drolet R, Higgins R, Messier R, Yan L, Coleman BE, Paster BJ, Dewhirst FE. *Helicobacter canis* isolated from a dog liver and multifocal necrotizing hepatitis. J Clin Microbiol 1996;34:2479–2482.
12. Fox JG, Li X, Yan L, Cahill RJ, Hurley R, Lewis R, Murphy JC. Chronic proliferative hepatitis in A/JCr mice associated with persistent *H. hepaticus* infection: a model of *Helicobacter*-induced carcinogenesis. Infect Immun 1996;64:1548–1558.
13. Lin TT, Yeh CT, Wu CS, Liaw YF. Detection and partial sequence analysis of *Helicobacter pylori* DNA in the bile samples. Dig Dis Sci 1995;40:2214–2219.
14. Kawaguchi M, Saito T, Ohno H, Midorikawa S, Sanji T, Handa Y, Morita S, Yoshida H, Misaka R, Hirota T, Saito M, Minami K. Bacteria closely resembling *Helicobacter pylori* detected immunohistologically and genetically in resected gall bladder mucosa. J Gastroenterol 1996;31: 294–298.
15. Ames WR, Robins M. Age and sex as factors in the development of the typhoid carrier state, and a method for estimating carrier prevalence. Am J Public Health 1943;33:221–230.
16. Welton JC, Marr JS, Friedman SM. Association between hepatobiliary cancer and typhoid carrier state. Lancet 1979;1:791–794.
17. Nomura A, Stemmerman GN, Chyou P-H, Pérez-Pérez GI, Blaser MJ. *Helicobacter pylori* infection and the risk for duodenal and gastric ulceration. Ann Intern Med 1994;120:977–981.
18. Cover TL, Berg DE, Blaser MJ. VacA and the *cag* pathogenicity island of *H. pylori*. In: Ernst PB, Michetti P, Smith PD, eds. The immunobiology of *H. pylori:* from pathogenesis to prevention. Philadelphia: Lippincott-Raven, 1997:75–90.
19. Polk BF, Kasper DL. *Bacteroides fragilis* subspecies in clinical isolates. Ann Intern Med 1977;86:569–571.
20. Blaser MJ. Microbial causation of the chronic idiopathic inflammatory bowel diseases. Inflamm Bowel Dis 1997;3:225–229.
21. Blaser MJ. Bacteria and diseases of unknown cause. Ann Intern Med 1994;121:144–145.

Address requests for reprints to: Martin J. Blaser, M.D., Division of Infectious Diseases, Vanderbilt University School of Medicine, A-3310 Medical Center North, 1161 21st Avenue South, Nashville, Tennessee 37232-2605. e-mail:martin.blaser@mcmail.vanderbilt.edu; fax: (615) 343-6160.

0016-5085/98/$3.00

Steatohepatitis: A Tale of Two "Hits"?

See article on page 764.

It has long been recognized that hepatic steatosis (fatty liver) occurs frequently in heavy alcohol drinkers and obese individuals.[1,2] It may also follow the ingestion of a wide variety of therapeutic drugs.[3] Steatosis of any etiology can be associated with the development of necroinflammation and fibrosis, so-called steatohepatitis, and even cirrhosis.[4] Furthermore, steatohepatitis caused by alcohol, drugs, or other forms of nonalcoholic steatohepatitis (NASH) share many histological features.[5] The question is whether these disparate causes could lead to steatohepatitis and its potential sequelae of cirrhosis or liver failure by one or more common mechanisms. Any satisfactory unifying mechanism should ideally explain why, in some individuals, steatosis, whatever its etiology, never progresses to steatohepatitis,[6] and also explain the variable incidence and severity of steatohepatitis and fibrosis in fatty liver of different etiologies. In this issue, Berson et al., working in Pessayre's laboratory, provide persuasive evidence that at least one such mechanism linking steatosis to necroinflammation and fibrosis is lipid peroxidation.[7]

A growing body of evidence supports a role for lipid peroxidation in the pathogenesis of alcohol-induced hepatitis and fibrosis.[8,9] Ethanol metabolism results in the formation of reactive oxygen species (ROS) and carbon-centered free radicals capable of initiating peroxidation of the polyunsaturated fatty acid side chains of membrane phospholipids and lipoproteins. Potential sources of free radicals are the ethanol-inducible cytochrome P450 2E1 (CYP2E1), which generates superoxide, hydroxyl, and hydroxyethyl radicals, the mitochondrial respiratory chain (in response to the increased ratio of reduced to oxidized nicotinamide adenine dinucleotide [NADH/NAD]), xanthine and aldehyde oxidases, and peroxisomal β-oxidation of free fatty acids, which generates hydrogen peroxide.[10] CYP2E1-mediated generation of hydroxyethyl radicals in particular correlates closely with the degree of lipid peroxidation and liver damage in animal models of alcoholic liver disease (ALD).[11] A role for lipid peroxidation in NASH has been suggested by recent studies showing its presence in both animal models of nonalcoholic fatty liver and humans with steatosis of different etiologies.[12–14]

A major attraction of lipid peroxidation as an important common pathogenic mechanism of steatohepatitis associated with fatty liver of different etiologies is that it potentially explains most, if not all, of the diverse histological features observed in this condition. Peroxidation of membrane lipids may cause cell necrosis and megamitochondria. The aldehyde products of lipid peroxidation, 4-hydroxynonenal and malondialdehyde (MDA), are capable of activating hepatic stellate cells,[15,16] the principal collagen-producing cells within the liver, cross-linking cytokeratins to form Mallory bodies,[12] and stimulating neutrophil chemotaxis.[17] MDA may also contribute to inflammation by activating NF-κB,[18] a transcription factor regulating the expression of several proinflammatory cytokines and adhesion molecules including tumor necrosis factor α, interleukin 8, intercellular adhesion molecule 1, and E-selectin.[19]

Previous work from Pessayre's group has suggested that the mere presence of oxidizable fat within the liver is enough to trigger lipid peroxidation.[12] However, many patients with steatosis never progress to necroinflammation or fibrosis.[6] This suggests that, in addition to steatosis (the first "hit"), the development of steatohepatitis requires the presence of some other factor(s) (second "hit"). This factor(s) might be expected to be particularly apparent in conditions where steatohepatitis is most commonly associated with steatosis, including alcohol-related liver disease and certain drugs. The results presented by Berson et al. show elegantly that one such second hit is a source of free radicals capable of inducing oxidative stress.

Many drugs are associated with the development of steatosis, including antiviral agents (interferon, zidovudine), aspirin, and other nonsteroidal anti-inflammatory drugs, the antiepileptic sodium valproate, and the tetracycline group of antibiotics. The fat is typically microvesicular in distribution and results predominantly from the inhibition of mitochondrial β-oxidation of fatty acids by a variety of different mechanisms.[3] However, only one class of drugs is commonly associated with classical steatohepatitis, the cationic amphiphilic amines: amiodarone, perhexiline, and the coronary dilator 4,4'-diethylaminoethoxyhexestrol (DEAEH).[3,20,21] Pessayre's group has shown previously that amiodarone[22,23] and perhexiline[24] accumulate in the mitochondria and inhibit not only β-oxidation (causing steatosis, the first hit) but also the transfer of electrons along the respiratory chain. Theoretically, this latter effect would be expected to generate superoxide anions capable of initiating lipid peroxidation,[25] thus providing a potential mechanism for the second hit required for steatohepatitis to develop. In this latest study they first show that, like amiodarone and perhexiline, DEAEH inhibits both β-oxidation and respiration, and then go on to show that all three drugs increase the production of ROS by isolated mitochondria and increase in vivo lipid peroxidation 5–10-fold. These data strongly suggest that it is because these drugs not only cause steatosis but also provide a mechanism for increased oxidative stress that they are capable of inducing steatohepatitis-like lesions.

This concept of steatohepatitis as a double-hit lesion potentially explains its relatively common occurrence in alcohol-related liver disease, where there exist several mechanisms for both steatosis[1] and oxidative stress. This theory may also explain why it has been difficult to develop animal models of alcoholic hepatitis and cirrhosis. In addition to simple ethanol administration, necroinflammation and fibrosis have only been produced by manipulations that have provided an alternative or extra source of oxidative stress. These have included increasing the dietary content of polyunsaturated fat (which induces the activity of CYP2E1[26]) and iron[27] (which favors the production of hydroxyl radicals from hydrogen peroxide) and coadministering carbon tetrachloride vapor.[28] Because most heavy drinkers develop steatosis, the apparent individual susceptibility to advanced ALD seems likely to be explained by interindividual differences in the magnitude of the second hit, oxidative stress. This may be genetically determined, e.g., possession of the more transcriptionally active c2 allele of CYP2E1,[29] or influenced by environmental factors such as dietary intake of anti- or pro-oxidants.

The work of Pessayre's group might also be extrapolated to steatohepatitis of non–drug-related or ethanol-related etiologies. In particular, the data might explain why NASH occurs more commonly in association with some causes of steatosis than others and with greater severity. Some degree of lipid peroxidation can be shown in steatosis of most etiologies.[12] This presumably reflects the mild level of oxidative stress arising from normal physiological processes and is insufficient to cause significant liver injury. By analogy with drug-induced steatohepatitis, for NASH to occur, some additional source of oxidative stress (the second hit) is required that is capable of initiating enough lipid peroxidation to overcome the normal cellular defense mechanisms and produce necroinflammation. Recent studies have suggested several potential sources for this second hit. Increased expression of CYP2E1 has been shown in patients and animal models of NASH.[30,31] In the absence of ethanol, CYP2E1 can generate free radicals from endogenously produced ketones and aldehydes and dietary *N*-nitrosamines. Possible

mediators of its induction in nonalcoholics include ketones and/or fatty acids,[32] both of which may explain the induction of CYP2E1 by a high-fat diet.[26] Interestingly, among patients with obesity-related steatosis, the risk factors for steatohepatitis/fibrosis include rapid weight loss caused by dieting, debilitation or intestinal bypass surgery, surgical stress, alcohol intake, and diabetes, all of which are associated with an increase in the concentration of fatty acids and/or ketones within the liver.[1] In addition to CYP2E1 induction, an increase in the intrahepatic concentration of free fatty acids may provide a further source of oxidative stress via peroxisomal β-oxidation. This pathway becomes important in conditions of substrate overload or when mitochondrial β-oxidation is inhibited.[33] Unlike its mitochondrial counterpart, peroxisomal β-oxidation produces hydrogen peroxide that, in the presence of free iron, is converted to the highly reactive hydroxyl radical. The importance of liver iron in disease pathogenesis has been illustrated by a recent study showing that patients with NASH have an increased frequency of the C282Y mutation in the recently cloned hemochromatosis gene, HFE.[34] Clearly, as for ALD, other genetic determinants of oxidative stress could also play a role in susceptibility to NASH.

This study by Berson et al. of a small group of drugs has provided a basis for understanding at least one of the important mechanisms of steatohepatitis regardless of etiology. Its development requires a double hit, the first producing steatosis, the second a source of oxidative stress capable of initiating significant lipid peroxidation. This concept provides a rationale for both the treatment and prevention of disease progression in steatosis of alcoholic and nonalcoholic causes.

CHRISTOPHER P. DAY
OLIVER F. W. JAMES
Centre for Liver Research
Newcastle Upon Tyne, England

References

1. Day CP, Yeaman SJ. The biochemistry of alcoholic fatty liver. Biochim Biophys Acta 1994;1215:33–48.
2. Braillon A, Capron JP, Herve MA, Degott C, Quenum C. Liver in obsesity. Gut 1985;26:133–139.
3. Fromenty B, Pessayre D. Inhibition of mitochondrial beta-oxidation as a mechanism of hepatotoxicity. Pharmacol Ther 1995;67:101–154.
4. Bacon BR, Farahvash MJ, Janney CG, Neuschwander-Tetri BA. Nonalcoholic steatohepatitis: an expanded clinical entity. Gastroenterology 1994;107:1103–1109.
5. Diehl AM, Goodman Z, Ishak KG. Alcohollike liver disease in nonalcoholics. Gastroenterology 1988;95:1056–1062.
6. Teli MR, James OFW, Burt AD, Bennett MK, Day CP. The natural history of non-alcoholic fatty liver: a follow-up study. Hepatology 1995;22:1714–1719.
7. Berson A, De Beco V, Lettéron P, Robin MA, Moreau C, El Kahwaji J, Verthier N, Feldmann G, Fromenty B, Pessayre D. Steatohepatitis-inducing drugs cause mitochondrial dysfunction and lipid peroxidation in rat hepatocytes. Gastroenterology 1998;114:764–774.
8. Nordmann R, Ribiere C, Rouach H. Implication of free radical mechanisms in ethanol induced cellular injury. Free Radic Biol Med 1992;12:219–240.
9. Day CP. Is necroinflammation a prerequisite for fibrogenesis? Hepatogastroenterology 1996;43:104–120.
10. Misra UK, Bradford BU, Handler JA, Thurman RG. Chronic ethanol treatment induces H_2O_2 production selectively in pericentral regions of the liver lobule. Alcohol Clin Exp Res 1992;16:839–842.
11. Albano E, Clot P, Morimoto M, Tomasi A, Ingelman-Sundberg M, French SW. Role of cytochrome P4502E1–dependent formation of hydroxyethyl free radical in the development of liver damage in rats intragastrically fed with ethanol. Hepatology 1996;23:155–163.
12. Letteron P, Fromenty B, Terris B, Degott C, Pessayre D. Acute and chronic hepatic steatosis lead to in vivo lipid peroxidation in mice. J Hepatol 1996;24:200–208.
13. Weltmann MD, Liddle C, Farrell GC. Evidence that oxidative stress plays a role in steatohepatitis produced in a rat nutritional model (abstr). Hepatology 1996;24:240A.
14. Pinto HC, Felipe P, Baptista A, Fernandes A, Camilo E, Ramalho F, Moura MC. Hepatic steatosis and lipid peroxidation in human chronic liver diseases (abstr). Hepatology 1996;24:311A.
15. Parola M, Pinzani M, Casini A, Albano E, Poli G, Gentilini A, Gentilini P, Dianzani MU. Stimulation of lipid peroxidation or 4-hydroxynonenal treatment increases procollagen alpha 1(I) gene expression in human liver fat-storing cells. Biochim Biophys Res Commun 1993;194:1044–1050.
16. Lee KS, Buck M, Houglum K, Chojkier M. Activation of hepatic stellate cells by TGF alpha and collagen type I is mediated by oxidative stress through c-myb expression. J Clin Invest 1995;96:2461–2468.
17. Curzio M, Esterbauer H, Dianzani MU. Chemotactic activity of hydroxyalkenals on rat neutrophils. Int J Tissue React 1985;7:137–142.
18. Jaeschke H, Wang Y, Essani NA. Reactive oxygen species activate the transcription factor NF-κB in the liver by induction of lipid peroxidation (abstr). Hepatology 1996;24:238A.
19. Baeuerle PA, Henkel T. Function and activation of NF-κB in the immune system. Annu Rev Immunol 1994;12:141–179.
20. Pessayre D, Bichara M, Feldmann G, Degott C, Potet F, Benhamou J-P. Perhexiline-maleate induced cirrhosis. Gastroenterology 1979;76:170–177.
21. Simon JB, Manley PN, Brien JF, Armstrong PW. Amiodarone hepatotoxicity simulating alcoholic liver disease. N Engl J Med 1984;311:167–172.
22. Fromenty B, Fisch C, Labbe G, Degott C, Deschamps D, Berson A, Letteron, P, Pessayre D. Amiodarone inhibits the mitochondrial β-oxidation of fatty acids and produces microvesicular steatosis of the liver in mice. J Pharmacol Exp Ther 1990;255:1371–1376.
23. Fromenty B, Fisch C, Berson A, Letteron P, Larrey D, Pessayre D. Dual effect of amiodarone on mitochondrial respiration, initial protonophoric uncoupling effect followed by inhibition of the respiratory chain at the levels of complex I and complex II. J Pharmacol Exp Ther 1990;255:1377–1384.
24. Deschamps D, De Beco V, Fisch C, Fromenty B, Guillouzo A, Pessayre D. Inhibition by perhexiline of oxidative phosphorylation and the β-oxidation of fatty acids: possible role in pseudoalcoholic lesions. Hepatology 1994;19:948–961.
25. Garcia-Ruiz C, Colell A, Morales A, Kaplowitz N, Fernandez-Checa JC. Role of oxidative stress generated from mitochondrial electron transport chain and mitochondrial electron status in loss of mitochondrial function and activation of transcription factor nuclear

factor-κB: studies with isolated mitochondria and rat hepatocytes. Mol Pharmacol 1995;48:825–834.
26. Takahashi H, Johansson I, French SW, Ingelman-Sundberg M. Effects of dietary fat composition on activities of the microsomal ethanol oxidizing system and ethanol-inducible cytochrome P-450 (CYP 2E1) in the liver of rats chronically fed ethanol. Pharmacol Toxicol 1992;70:347–351.
27. Tsukamoto H, Horne W, Kamimura S, Niemela O, Parkkila S, Yla-Herttuala S, Brittenham G. Experimental liver cirrhosis induced by alcohol and iron. J Clin Invest 1995;96:620–630.
28. Hall P, Plummer J, Ilsley A, Cousins M. Hepatic fibrosis and cirrhosis after chronic administration of alcohol and "low-dose" carbon tetrachloride vapour in the rat. Hepatology 1991;13:815–819.
29. Grove J, Daly AK, Brown ASUM, Bassendine MF, James OFW, Day CP. The *Rsa* I polymorphism of CYP2E1 and susceptibility to alcoholic liver disease in Caucasians: effect on age of presentation and dependence on alcohol dehydrogenase genotype. Pharmacogenetics (in press).
30. Weltmann MD, Liddle C, Farrell GC, Hepatic CYP2E1 is increased in patients with non-alcoholic steatohepatitis (abstr). Hepatology 1996;24:202A.
31. Weltmann MD, Farrell CG, Liddle C. Increased CYP2E1 expression in a rat nutritional model of hepatic steatosis with inflammation. Gastroenterology 1996;111:1645–1653.
32. Zangar RC, Novak RF. Effects of fatty acids and ketone bodies on cytochromes P450 2B, 4A, and 2E1 expression in primary cultured rat hepatocytes. Arch Biochem Biophys 1997;337:217–224.
33. Ockner RK, Kaikus RM, Bass NM. Fatty-acid metabolism and the pathogenesis of hepatocellular carcinoma: review and hypothesis. Hepatology 1993;18:669–676.
34. George DK, Goldwurm S, Macdonald GA, Cowley LL, Walker NI, Ward PJ, Jazwinska EC, Powell LW. Increased hepatic iron concentration in nonalcoholic steatohepatitis is associated with increased fibrosis. Gastroenterology 1998;114:311–318.

Address requests for reprints to: Christopher P. Day, M.D., Ph.D., Centre for Liver Research, Floor 4, William Leech Building, Medical School, Framlington Place, Newcastle Upon Tyne, England. Fax: (44) 191-222-0723.

0016-5085/98/$3.00